Molecular Therapy
Nucleic Acids
Commentary

MRNACalc: An accurate RNA quantification tool in the era of modified nucleosides

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With the advent of COVID-19 vaccines, the utilization of messenger RNA (mRNA) has experienced a remarkable surge, driving the development of novel vaccines and therapeutics against a range of infectious and non-infectious diseases. The incorporation of modified nucleosides such as pseudouridine (Ψ), N1-methylpseudouridine ($m^1\Psi$), and 5-methylpseudoruridine (m^5C) into mRNA molecules has emerged as a strategy to enhance their efficacy by reducing cytoplasmic RNA sensor activation and improving translation efficiency, a discovery for which the Nobel prize was awarded to Drew Weissmann and Katalin Karikó. However, the introduction of these modified nucleotides has complicated the accurate measurement of mRNA concentrations using ultraviolet (UV) spectrophotometric methods. In their recent work, Finol and colleagues developed the mRNACalc web server, a web-based tool for accurate quantification of RNA containing modified nucleosides.[1] This tool enables the refinement of RNA concentration measurements, thus facilitating accurate dosing strategies essential for future clinical applications (Figure 1).

The remarkable success of COVID-19 mRNA vaccines forms the culmination of a long journey that began in 1961 when Sydney Brenner, Francois Jacob, and Matthew Meselson discovered mRNA in bacteria.[2,3] This journey progressed to successful preclinical experiments in 1990 by Wolff et al., who demonstrated for the first time that mRNA can be used for *in vivo* gene transfer.[4] Building upon this foundation, Katalin Karikó and Drew Weissman discovered, in early 2000, that the incorporation of modified nucleosides into mRNA could mitigate its intrinsic immunostimulatory activity while preserving translation efficiency.[5–7] While these modifications paved the way for the success of today's mRNA-based vaccines, they have also introduced complexities to RNA quantification.

To address these challenges, Finol and colleagues designed mRNACalc, a web tool that accurately quantifies mRNA while taking into account its composition.[1]

Accurate determination of RNA concentration and purity is crucial for downstream applications, especially in the development of mRNA-based vaccines. To date, UV spectrophotometry remains the preferred method for mRNA quantification due to its simplicity, cost-effectiveness, and minimal requirements for sample preparation. This technique involves measuring the UV absorbance of RNA at a wavelength of 260 nm (A260) and subsequently applying the Beer-Lambert law ($A = \varepsilon \cdot C \cdot l$), where ε represents the molar absorption coefficient, A is the measured absorbance, C denotes the concentration of the compound, and I is the optical pathlength. However, the accuracy of this method relies on factors such as the extinction coefficient (ε) or molar absorption coefficient specific to the analyzed material.[8]

In a recent study, Nwokeoji et al. underscored the importance of hypochromicity in oligonucleotide or complex nucleic acid.[9] Incorporating modified nucleosides complicates RNA quantification by altering the spectrophotometric properties of the RNA. In the current study, the authors validated the hypochromic effect of modified nucleosides by comparing the MAC260 of standard nucleosides such as uridine (U) and cytidine (C) with their modified counterparts Ψ, $m^1\Psi$, and m^5C. Indeed, the MAC260 values of each modified nucleoside were reduced, indicating that chemical modifications of these pyrimidine nucleotides affected the spectrophotometric properties, resulting in an underestimation of the mRNA concentration. Furthermore, RNA hypochromicity is affected by the sequence folding of RNA and the chemical environment of the nucleobases. To achieve a more precise quantification, RNA is often subjected to thermal and alkaline hydrolysis before UV measurement, which transitions the hypochromic folded state of RNA to the hyperchromic state of individual monophosphate nucleotides. The combination of both hypochromic correction and hydrolysis of RNA forms the basis of the mRNACalc web tool. This web tool was validated using both traditional UV spectroscopy and a fluorescence-based assay (Ribogreen). While the latter method offers a more sensitive approach, the binding affinity of the modified nucleosides to the dye may be potentially affected. Indeed, both traditional methods exhibited significant underestimation of the measured concentration in comparison with the mRNACalc web tool.

The rapid roll-out of the COVID-19 mRNA vaccines amid the pandemic hindered researchers from conducting comprehensive investigations into optimizing RNA concentration measurement. Given that the efficacy and safety of mRNA vaccines are dose dependent, ongoing refinement and the identification of an accurate method for RNA quantification are fundamental. With our enhanced comprehension of how nucleoside modifications impact RNA concentration determination, researchers can now achieve more accurate measurements utilizing the mRNACalc tool. While this web tool represents a significant advancement in conventional, non-replicating mRNA quantification, it does not accommodate

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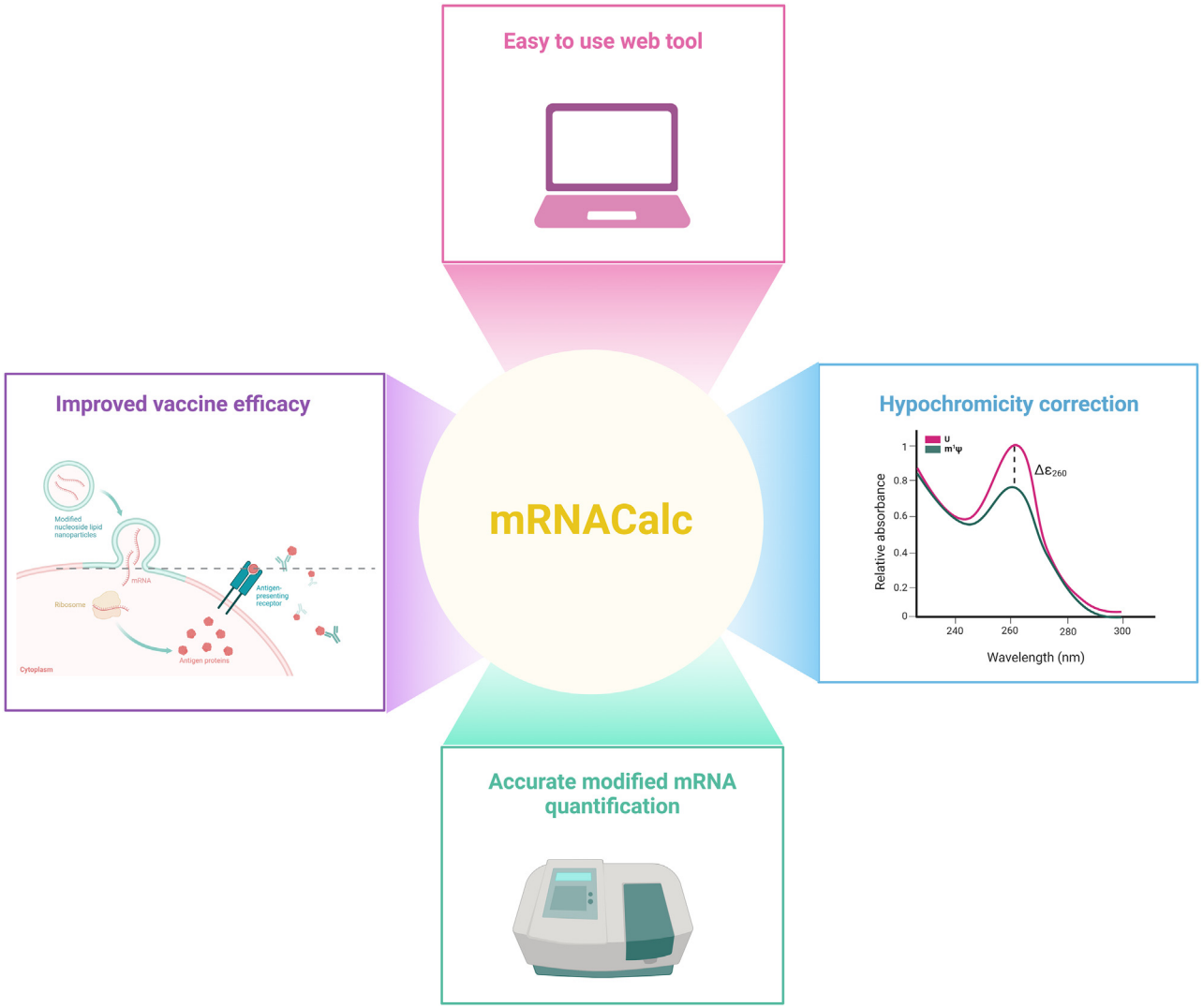

Figure 1. Advantages of using mRNA Calc to quantify nucleoside modified RNA
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complex RNA compositions such as self-amplifying RNAs and more recently *trans*-amplifying RNAs, which pose additional challenges due to their large sequences and self-amplification capacity. Furthermore, although the authors demonstrated an underestimation of RNA quantification, further research is required to compare the *in vitro* and *in vivo* efficacy as well as immunogenicity of the mRNA quantified using the mRNACalc web tool with that of traditional methods. Additionally, it would be valuable to explore whether this tool could determine the encapsulation efficiency of formulated RNA and whether various RNA formulations might influence RNA quantification.

Taken together, Finol and colleagues successfully designed the RNA quantification tool mRNACalc. This user-friendly web tool will undoubtedly play a crucial role in the development of novel mRNA therapeutics, paving the way for enhanced efficacy and safety in future applications.

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DECLARATION OF INTERESTS

There are no conflicts of interest to disclose.

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